Remarks

Reconsideration of this Application is respectfully requested.

Claims 2-5 and 9-10 are pending in the application.

Claim 9 stands rejected and claim 10 has been objected to.

Based on the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

Allowance of Claims 2-5

Applicants thank the Examiner for indicating that claims 2-5 are allowable. (Paper No. 30, page 1.)

Objection to Claim 10

The Examiner has indicated that claim 10 is objected to. (Paper No. 30, page 1.) However, the Examiner has not stated the basis for this objection. As the Examiner has failed to substantiate the objection, Applicants respectfully request that the objection be reconsidered and withdrawn.

Rejection under 35 U.S.C. § 102

The Examiner has rejected claim 9 under 35 U.S.C. § 102(a) or § 102(b) as allegedly anticipated by Kim *et al.*, *J. Neurobiology 17*: S155 (1996). (Paper No. 30, page 2.) In support of the rejection, the Examiner has stated that:

> Kim et al., teaches a method of screeninfg [sic] a compound, tetracycline or cyclohexamide, wherein said compound decreases the amount of the 20kDA PS2-CTF fragment in a cell and compares it to a control.
>
> The prior art teachings anticipate the claimed invention. It is noted that the rejection is a 102(a) or (b) since the exact date the abstract book was made publicly available has yet to be determined. When the date is made available, it will be communicated to Applicant.

(Paper No. 30, page 2.) Applicants respectfully traverse the rejection.

As evidenced from the document itself, the Kim *et al.* publication is the abstract of research presented at the Fifth International Conference on Alzheimer's Disease. This conference was held July 24-26, 1996 in Osaka, Japan. *See* Exhibit A. Applicants have spoken with one of the event coordinators of the conference, Khalid Iqbal, who has indicated that the abstracts were not distributed to the participants of the conference until the participants arrived at the conference site. Therefore, July 24, 1996 is the earliest date in which the Kim *et al.* publication was made publicly available. As the instant application claims priority to a provisional application which was filed on April 24, 1997, Kim *et al.* was not published "more than one year prior to the date of the application for patent. . . ." 35 U.S.C. § 102(b). Accordingly, Kim *et al.* is not 35 U.S.C. § 102(b) art and does not anticipate claim 9.

Further, in accordance with MPEP § 715.01(c) (Rev. August 2001), submitted herewith is a copy of a Declaration of Co-Inventors Under 37 C.F.R. § 1.132 ("132 Declaration"), executed by Drs. Rudolph Tanzi and Tae-Wan Kim. This 132 Declaration was original filed on July 31, 2000 in the captioned application. For the convenience of the Examiner, a copy of the 132 Declaration is submitted herewith along with a postcard indicating receipt of the 132 Declaration in the USPTO. In the 132 Declaration, Drs. Tanzi and Kim establish that the work disclosed in Kim *et al.* is their own and that the other co-authors of Kim *et al.* were merely working under Drs. Tanzi and Kim's direction, providing technical assistance, or providing a reagent used in the experiments. Thus, the 132 Declaration is sufficient to remove Kim *et al.* as a reference under 35 U.S.C. § 102(a) as the work described in Kim *et al.* is the inventors own work and the other co-authors of Kim *et al.* are not co-inventors of the subject matter of the captioned application.

In view of the above, Applicants assert that Kim *et al.* is neither 35 U.S.C. §102(a) nor § 102(b) art. Applicant, therefore, respectfully request that the Examiner reconsider and withdraw the rejection of claim 9.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance.

If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Reply is respectfully requested.

Respectfully submitted,

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FIFTH INTERNATIONAL CONFERENCE

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Fifth International Conference on Alzheimer's Disease and Related Disorders

24-29 July, 1996 Osaka, Japan

More than 1500 people have registered to attend this meeting, making it the largest ever convened in the field of Alzheimer's disease research. The Alzheimer Research Forum marks the public launch of its pilot World Wide Web site by featuring the following 12 presentations from the meeting. The recorded presentations will be edited into virtual slide talks and will be available on the site from around August 1. The presenters have agreed to respond to questions from the Web audience for an eight-week period after the conference.

The audio portions of the following presentations are in Real Audio format. To listen to Real Audio you will need to download and install the RealOne Player. This version of the RealOne Player is free.

- **Synthesis and metabolism of presenilin 1 with mutations linked to familial Alzheimer's Disease in transgenic mice.**
  David Borchelt (Sangram Sisodia presenting), Johns Hopkins Medical Institutions.
  (#95 (poster), 24 July, 16:00-18:00.)

- **Evolution of Alzheimer's disease related intraneuronal changes.**
  Heiko Braak, J.W. Goethe University.
  (#144 (plenary), 25 July 8:30-9:30.)

- **The presenilin 1 and 2 mutations linked to familial Alzheimer's disease increase the extracellular concentration of amyloid beta protein ending at AB42(43).**
  Steven Younkin, Mayo Clinic Jacksonville.
  (#149 (symposium), 25 July, 11:20-11:40.)

- **Complete genomic screen in late-onset familial Alzheimer's disease.**
  Margaret Pericak-Vance, Duke University Medical Center.
  (#233 (poster), 25 July, 16:00-18:00.)

- **Involvement of tau protein kinase in Alzheimer's disease pathogenesis.**

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Kazutomo Imahori, Mitsubishi Kasei Institute of Life Sciences.
(#300 (symposium), 26 July, 11:20-11:40.)

- **Estrogen, ApoE and the risk of Alzheimer's disease**.
  Cornelia Van Duijn, Erasmus University Medical School. (#315 (oral), 26 July, 12:00-12:15.)

- **Anti-inflammatory drug use and risk of incident Alzheimer's disease in a defined community population**.
  Laurel A. Beckett, Rush University.
  (#318 (oral), 26 July, 12:45-13:00.)

- **Cholinergic function and apoE polymorphism in Alzheimer's disease**.
  Hilkka Soininen, University of Kuopio.
  (#540 (poster), 27 July, 9:30-11:30.)

- **Two homologous genes associated with early onset familial Alzheimer's disease**.
  Peter St. George-Hyslop, University of Toronto.
  (#610 (symposium), 28 July, 12:00-12:20.)

- **Senile dementia with abundant neurofibrillary tangles without accompanying senile plaques. A new disease entity separable from SDAT?**
  Kenji Ikeda, Tokyo Institute of Psychiatry.
  (#605 (symposium), 28 July, 11:20-11:40.)

- **Proteolytic Processing of Wild-Type and Mutant Forms of Presenilin 2**.
  Tae-Wan Kim, Massachusetts General Hospital and Harvard Medical School.
  (#624 (oral), 28 July, 12:30-12:45.)

- **Monokines Reduce Accumulatoin of Beta-Peptide in Cultured Vascular Smooth Muscle Cells by Enhancing the Nonamyloidogenic Processing of Beta-APP**.
  Janusz Frackowiak, New York State Institute for Basic Research in Developmental Disabilities.
  (#797 (oral), 29 July, 11:15-11:30.)

Biochem. J. (1995) 310, 337–343 (Printed in Great Britain)

Development of a cleavage-site-specific monoclonal antibody for detecting metalloproteinase-derived aggrecan fragments: detection of fragments in human synovial fluids

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We have developed a monoclonal antibody AF-28 that specifically recognizes a neo-epitope on polypeptides with N-terminal FFGVG... sequences. This sequence is found at the N-terminus of aggrecan fragments that have been digested with matrix metalloproteinases (MMPs). By immunoblotting, monoclonal antibody AF-28 specifically detected G2 fragments derived from an aggrecan G1–G2 substrate digested with stromelysin, collagenase, gelatinase and matrilysin, but failed to detect G2 fragments obtained from elastase, trypsin or cathepsin B digests. Undigested G1–G2 was not detected. In addition, AF-28 antibody detected fragments derived from whole aggrecan and this detection did not require prior treatment with chondroitinase or keratanase. Competition experiments confirmed that peptides containing internal ...FFGVG... sequences were not detected by the antibody, while native MMP-digested aggrecan fragments and a synthetic 32-mer peptide with FFGVG... N-termini were equally competitive on a molar basis. An FFGVG 5-mer, and an FGVGGEEDI 9-mer which lacked the N-terminal phenylalanine residue, were 50 times and 230 times respectively less competitive than the FFGVG... 32-mer. Two fragments from the interglobular domain, F^{342}–E^{373} and F^{342}–D^{441}, that are predicted products of G1–G2 digestion by neutrophil collagenase but have not previously been detected, could be detected with AF-28. The epitope recognized by AF-28 was also detected in human synovial fluids by Western blot analysis. A broad band of 100–200 kDa was detected in some patients and a dominant band of 40–60 kDa was found in two patients. The size of this small fragment corresponds with that seen for the porcine F^{342}–E^{373} product and may represent the natural physiological product of aggrecan cleaved *in vivo* at both the MMP site (...$DIPEN^{341}$↓F^{342}FGVG...) and the aggrecanase site (...$ITEGE^{373}$↓A^{374}RGSVI...).

INTRODUCTION

Aggrecan is the major proteoglycan present in articular cartilage and is the molecule that endows cartilage with its intrinsic properties of load-bearing and resisting compressive forces. These properties are critically dependent upon maintaining the structure and integrity of collagen and aggrecan within the extracellular matrix. Normal turnover of aggrecan is a conservative process in which the rate of breakdown and release of fragments from the tissue does not exceed the rate at which it is replaced by newly synthesized molecules. In pathology, the rate of degradation exceeds the rate of synthesis and there is a net loss of aggrecan, and the tissue becomes thin and mechanically weak.

The loss of aggrecan from cartilage during arthritic disease is the result of proteolytic attack on the core protein. The aggrecan core protein comprises two N-terminal globular domains, G1 and G2, and a C-terminal G3 globular domain. A long extended region of polypeptide between G2 and G3 is heavily substituted with chondroitin sulphate and keratan sulphate side chains and these represent almost 90% of the mass of aggrecan [1]. A short extended interglobular domain (IGD) between G1 and G2 is a major site of proteolysis, and the matrix metalloproteinases (MMPs) are known to cleave in this region *in vivo* [2,3] and *in vitro* [4–6]. The MMPs constitute a family of important extracellular proteinases and their activities in cartilage appear to be complex and highly regulated at both the intracellular and the extracellular levels. They are induced in the presence of certain stimuli, secreted as proenzymes that require activation, and their extracellular activities are controlled by specific tissue inhibitors.

Earlier studies showed that aggrecan turnover involved cleavage near the G1 domain to release large glycosaminoglycan-containing fragments that did not contain G1 and were unable to to bind hyaluronan [7–9]. More recently, proteinase cleavage sites within the IGD have been identified at the molecular level [4–6,10–16]. Our studies have identified cleavage sites specific for MMP-1 (fibroblast collagenase), MMP-2 (gelatinase A), MMP-3 (stromelysin-1), MMP-7 (matrilysin), MMP-8 (neutrophil collagenase) and MMP-9 (gelatinase B) [4–6] as well as cathepsin B [5], plasmin and urokinase-type plasminogen activator [10]. The results show that all the MMPs cleave at a site located between N^{341} and F^{342}, based on the human aggrecan sequence [17]. Thus N^{341}–F^{342} represents the preferred and predominant site of cleavage for this class of enzyme.

In other studies, cartilage explant cultures have been shown to release aggrecan fragments with N-terminal sequences corresponding to cleavage between E^{373} and A^{374} [11–13]. This site is located 32 amino acids C-terminal to the MMP cleavage site. In addition, the major aggrecan fragments found in synovial fluids from osteoarthritis and joint injury patients result from cleavage at the same E^{373}–A^{374} bond [15,16]. The enzyme responsible for this cleavage has been named 'aggrecanase', and although MMP-8 exhibits aggrecanase activity *in vitro* [18], the identity of the aggrecanase enzyme present in cartilage remains unknown. The occurrence of C-terminal ...$DIPEN^{341}$ fragments in cartilage

Abbreviations used: IGD, interglobular domain; MMP, matrix metalloproteinase; ABTS, 2,2'-azinodi-(3-ethylbenzthiazoline sulphonate); Fmoc, fluoren-9-ylmethoxycarbonyl; KLH, keyhole limpet haemocyanin; AEBSF, [4-(2-aminoethyl)benzene]sulphonyl fluoride.

‡ To whom correspondence should be addressed.

matrix [2,3] and N-terminal A^{374}RGSVI... fragments in joint fluids [15,16] suggests that cleavage at both the MMP site and the aggrecanase site are involved in aggrecan degradation *in vivo*.

Antibodies that specifically recognize newly created N- or C-termini resulting from proteolytic cleavage (neo-epitopes) have been described previously [19–21] and offer enormous potential for identifying products of discrete catabolic events. In this paper we report the development of a new monoclonal antibody that specifically recognizes the N-terminal FFGVG... sequence generated by MMP digestion of aggrecan. We have used this antibody to monitor MMP action against aggrecan in experimental systems and to detect the presence of MMP-degraded aggrecan in human synovial fluids. AF-28 antibody specifically identified small IGD fragments that had previously been predicted but not detected. Our results show that AF-28 epitopes are present in human synovial fluids, suggesting that MMPs are involved in the degradation of aggrecan *in vivo*.

EXPERIMENTAL

A chemiluminescence Western blotting kit, 2,2′-azinodi-(3-ethylbenzthiazoline sulphonate) (ABTS), cysteine proteinase inhibitor E-64 and pepstatin were from Boehringer, Mannheim, Germany. Keratanase (*Pseudomonas* sp.) (EC 3.2.1.103), keratanase II (*Bacillus* sp.) and chondroitin ABC lyase (*Proteus vulgaris*) (EC 4.2.2.4) were from Seikagaku, Kogyo, Japan. Trypsin (diphenylcarbamoyl-treated) (EC 3.4.21.4), soya bean trypsin inhibitor, keyhole limpet haemocyanin (KLH), 6-maleimidohexanoic acid *N*-hydrosuccinimide, 4 aminophenylmercuric acetate, 1,10-phenanthroline and hyaluronidase (*Streptomyces hyalurolyticus*) type IX were from Sigma. Elastase purified from human neutrophils was from ICN Biochemicals. Agarose type HSC was from PS Park Scientific (Northampton, U.K.). [4-(2-Aminoethyl)benzene]sulphonyl fluoride (AEBSF) was from Calbiochem-Novabiochem. Freund's complete adjuvant was from CSL Ltd. Rabbit anti-mouse horseradish peroxidase-conjugated immunoglobulin was from DAKO. Poly(vinylidene difluoride) membrane (Immobilon) was from Millipore–Waters. Methods for the preparation of the G1–G2 substrate have been described [22]. Native purified MMP-8 from human neutrophils [23] was generously provided by Professor Harald Tschesche, Bielefeld, Germany, and Dr. Vera Knäuper, Bielefeld, Germany. Recombinant human prostromelysin [24,25], recombinant human fibroblast procollagenase [26], recombinant human promatrilysin [27] and human progelatinase A [28] were prepared as described and were gifts from Dr. Gillian Murphy, Strangeways Laboratory, Cambridge, U.K. Recombinant rat cathepsin B was purified as described [29] and was generously provided by Dr. John Mort, Montreal, Canada.

Synthesis of peptide FFGVGGEEDC for elicitation of monoclonal antibody AF-28

The synthetic peptide FFGVGGEEDC was synthesized in a Crystal automatic synthesizer (Novabiochem) using Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry with counter-ion distribution monitoring enabled. Cleavage from the solid support was carried out in 90% (w/v) trifluoroacetic acid, 2% (w/v) anisole, 3% (w/v) ethanedithiol and 5% (w/v) thioanisole for 2 h at room temperature. Following partial evaporation of the peptide-containing filtrate under N_2, the crude peptide was precipitated in cold diethyl ether containing β-mercaptoethanol to maintain the cysteine residue in its reduced form. The crude peptide was purified by reverse-phase chromatography using a PepRPC 10/10 (C2/C18) column installed in an FPLC system (Pharmacia Pty. Ltd). The 9-mer peptide with sequence FGVGGEEDI was prepared by the same procedure.

Production of monoclonal antibody AF-28

The FFGVGGEEDC peptide was coupled to KLH carrier protein using the heterobifunctional agent 6-maleimidohexanoic acid *N*-hydrosuccinimide ester to form a peptide conjugate [30]. Balb/c mice were immunized with 75 μg of the conjugate emulsified in Freund's complete adjuvant and boosted with an equivalent dose 7 weeks later. Spleen cells from these mice were fused with SP_2O cells and hybrid cells were isolated using the 'HAT' selection procedure [31]. Culture fluids from the resulting hybridomas were screened against KLH and FFGVGGEEDC-KLH antigens by ELISA. Hybridomas positive against peptide conjugated to KLH, but not KLH alone, were cloned by limiting dilution. One hybridoma cell line, designated AF-28, was expanded in culture and used for ascites production. Antibody isotyping by ELISA determined that the subclass of the antibody was IgG1.

Enzyme digestions

MMP digestions were done in buffer containing 10 mM $CaCl_2$, 100 mM NaCl, 50 mM Tris/HCl, pH 7.5, at 37 °C and contained 1 mM AEBSF, 2 μM pepstatin and 20 μg/ml E-64 as proteinase inhibitors. The MMPs were activated as described previously [5,6]. Elastase and trypsin digests were carried out in 50 mM Tris/acetate buffer, pH 7.2, 10 mM EDTA at 37 °C. Cathepsin B digests were done in buffer containing 0.2 M NaCl, 1 mM EDTA, 10 mM dithiothreitol, 0.25 M sodium acetate, pH 5.5, at 37 °C. All G1–G2 samples were keratanase-treated prior to electrophoresis. Keratanase digests were done in 50 mM Tris/acetate buffer, pH 7.5, at 37 °C overnight with 0.025 unit of keratanase/30 μl, in the presence of 100 mM hexanoic acid, 5 mM benzamidine hydrochloride, 10 mM EDTA, 20 μg/ml E-64 and 1 mM AEBSF. Hyaluronidase digestion of human synovial fluids was done overnight at 37 °C by adding 15 μl of 400 units/ml enzyme to 1 ml of synovial fluid and adding concentrated stocks of proteinase inhibitors to bring to final concentrations of 10 mM EDTA, 1 mM AEBSF, 2 μM pepstatin and 20 μg/ml E-64. Precipitate in the digested samples was removed by centrifugation and the clear fluids were stored frozen.

Immunodetection with monoclonal AF-28 antibody

Samples electrophoresed on SDS/5% (w/v) polyacrylamide gels [32] or on agarose/acrylamide composite gels [33] were transferred on to Immobilon membrane and the membranes were blocked for 1 h at room temperature in 5% (w/v) skim milk powder in PBS. The blocked membranes were incubated with AF-28 antibody (1:2000 dilution) in 1% skim milk powder in PBS for 1 h at room temperature, washed six times in buffer containing 0.1% Tween-20 in PBS, then incubated for a further 1 h with an anti-mouse antibody conjugated with horseradish peroxidase (1:10000 dilution) at room temperature in 1% skim milk powder in PBS. After six washes in PBS/0.1% Tween-20, the membranes were treated with ECL reagent according to the manufacturer's instructions.

ELISA

Plastic microtitre plates (Immulon-4; Dynatech Laboratories Inc.) were coated with a 32-amino-acid synthetic peptide with the sequence FFGVGGEEDITVQTVTWPDMELPLPRNITEGE. This is part of the amino acid sequence present in the human

aggrecan IGD [17], where FFGVG ... represents the N-terminal sequence generated by the action of MMPs and ... NITEGE represents the C-terminal sequence generated by the action of an 'aggrecanase' enzyme. The peptide was prepared with free N- and C-termini on an Applied Biosystems 431A automated synthesizer using Fmoc chemistry. The crude peptide was purified on a C8 reverse-phase column and the structure was confirmed by electrospray mass spectrometry and from the amino acid analysis. (The FFGVG 5-mer and the DIPENFFGVG 10-mer were synthesized by the same procedure and both contained amidated C-termini.)

Plates were coated overnight at 4 °C with 200 μl of either 25 or 125 pmol/ml 32-mer peptide in 20 mM sodium carbonate, pH 9.6. The coating solution was removed and the plates were washed three times in a Tris incubation buffer (Tris IB) containing 0.1 % BSA, 0.1 % Nonidet P40, 0.15 M NaCl and 10 mM Tris/HCl, pH 7.4, then blocked for 1 h at 37 °C with 1 % BSA in Tris IB. After washing, samples or standard (100 μl) diluted in Tris IB were added to the wells, followed by 100 μl of AF-28 antibody diluted 1:1000 in Tris IB, and incubated for 1 h at 37 °C. The plates were washed and incubated with an anti-mouse antibody conjugated with horseradish peroxidase diluted 1:1000 in Tris IB, for 1 h at 37 °C. After washing, 200 μl of the peroxidase substrate ABTS was added and the plates were incubated at room temperature during colour development. Progress of the colour development was monitored at 405 nm, and when the absorbance in the wells containing antibody with no competitor (maximum binding) reached > 1.2 (10–30 min) the reaction was stopped by the addition of 25 μl of 10 % (w/v) SDS.

Collection of human synovial fluids

Human synovial fluids were obtained from the knee joints of patients attending Rheumatology Clinics who required either diagnostic or therapeutic aspiration. Complete aspirations of joint fluid were performed under aseptic conditions, without anaesthetic, using a 1.2 mm bore needle. The fluids were collected into sterile tubes, centrifuged within 1 h to remove cells and stored frozen at −20 °C.

RESULTS

Ascitic fluid containing the monoclonal antibody AF-28 was tested for immunoreactivity against a number of antigens by Western blot analysis and competition ELISA. To meet the requirement for a neo-epitope antibody it was essential that the antibody recognize only the N-terminal sequence of the immunizing peptide FFGVGGEEDC, and not an internal sequence. Thus the antibody should detect only aggrecan fragments containing the FFGVG ... N-terminal sequence produced by digestion with MMPs. It should not detect undigested aggrecan, nor aggrecan digested with enzymes that cleave at sites that are different to the major MMP site.

Detection of AF-28 epitope in MMP digests of G1–G2

A G1–G2 fragment isolated from pig laryngeal aggrecan [22] was digested with MMP-1, MMP-2, MMP-3, MMP-7 and MMP-8. G1–G2 was also digested with cathepsin B, elastase and trypsin, which do not cleave at the major MMP site [5,14]. Aliquots of the digests were analysed on gels in the presence of SDS and detected by silver staining (Figure 1a) or immunodetection with AF-28 (Figure 1b). The results show that AF-28 specifically detects products of MMP digestion, but not undigested G1–G2 or fragments produced by cathepsin B, elastase or trypsin digestion. A single AF-28-positive band of 85 kDa was found in the MMP-3, MMP-2 and MMP-7 digests, and this corresponds with the 85 kDa G2 band which has been isolated previously and shown to have an FFGVG ... N-terminus [18] The weak AF-28 staining seen in the MMP-7 digest (Figure 1b, lane 9) reflects the low level of enzyme activity in this particular experiment, and a significant

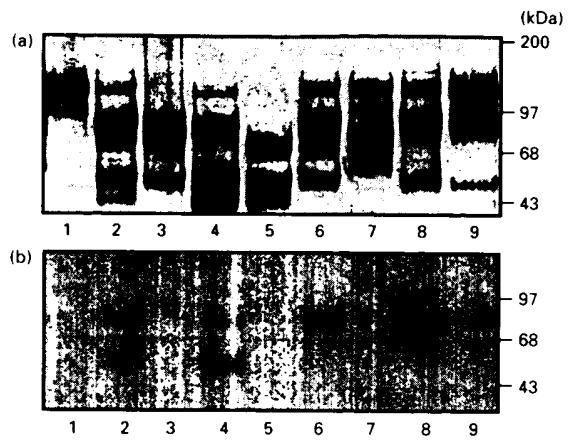

Figure 1 Proteinase digestion of aggrecan G1–G2 and immunodetection with AF-28

Purified aggrecan G1–G2 (5 μg) was digested with 125 μg/ml purified human MMP-8 (lane 2), 100 μg/ml recombinant rat cathepsin B (lane 3), 117 μg/ml recombinant human MMP-1 (lane 4), 0.1 unit/ml purified elastase (lane 5), 10 μg/ml recombinant human stromelysin (lane 6), 1 μg/ml trypsin (lane 7), 10 μg/ml purified human MMP-2 (lane 8) or 5.2 μg/ml recombinant human matrilysin. Lane 1 is an undigested sample. Following digestion with proteinases, the samples were treated with keratanase I prior to electrophoresis. Bands in the gels were detected (**a**) by silver stain or (**b**) by Western analysis with AF-28 monoclonal antibody.

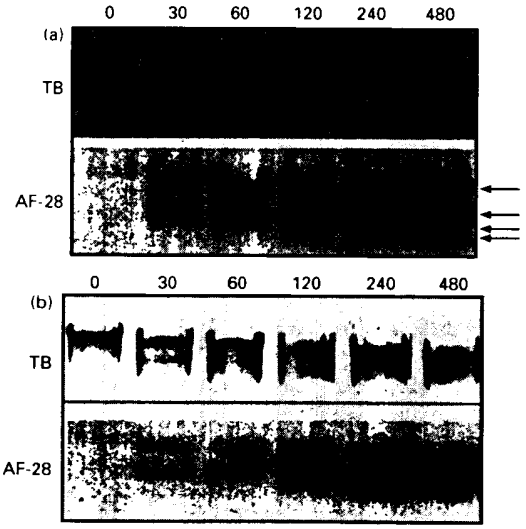

Figure 2 Different patterns of aggrecan fragments following collagenase digestion

Purified aggrecan was digested with 140 μg/ml purified human MMP-8 (**a**) or recombinant human MMP-1 (**b**) for 30, 60, 120, 240 or 480 min as indicated and the bands were revealed by Toluidine Blue (TB) staining and AF-28 immunodetection. The samples were not treated with keratanase. The first lane contains undigested aggrecan. The arrows indicate digestion products.

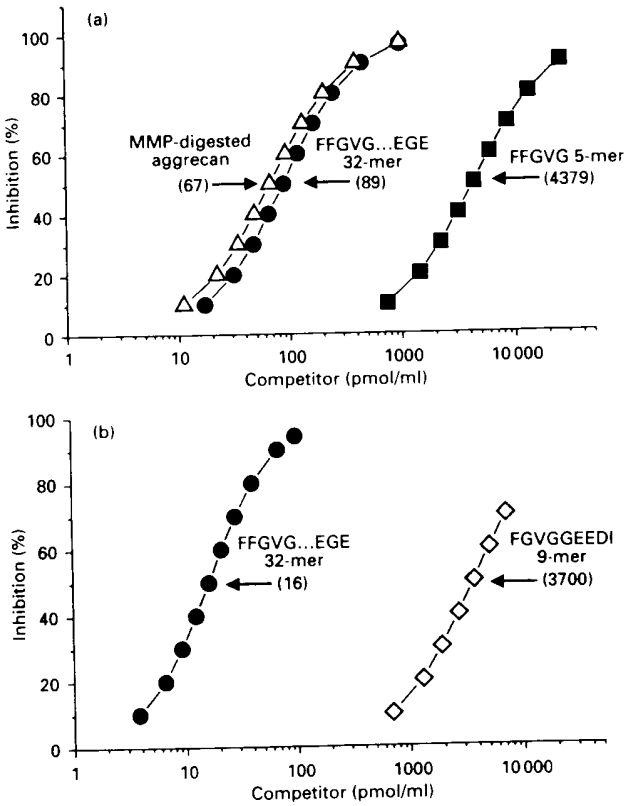

Figure 3 AF-28 competition experiments

ELISA plates (96-well) were coated with a synthetic FFGVG ... EGE 32-mer, at either (**a**) 125 pmol/ml (**b**) or 25 pmol/ml, and competition for binding of AF-28 to the coated antigen was tested with (**b**) FFGVG ... EGE 32-mer and FGVGGEEDI 9-mer or (**a**) FFGVG 5-mer, DIPENFFGVG 10-mer, FFGVG ... EGE 32-mer, undigested aggrecan and aggrecan digested with 140 μg/ml MMP-8. This concentration of MMP-8 completely degraded G1–G2 substrate, as determined by SDS/PAGE. Binding of AF-28 to the plate was detected with anti-mouse immunoglobulin conjugated to horseradish peroxidase, and colour development was measured at 405 nm after addition of the peroxidase substrate ABTS. The 50% inhibition values (pmol/ml) for each competitor are given in parentheses. Note that the sensitivity of the assay was improved approx. 5-fold by coating with 5 times less antigen. No data points are shown for undigested aggrecan or DIPENFFGVG, which did not compete in the assay.

amount of undigested G1–G2 can also be seen in the corresponding silver stain track (Figure 1a, lane 9). MMP-1 and MMP-8 produced two AF-28-positive bands, consistent with these enzymes cleaving G1–G2 at the major MMP site as well as at a second site (... $ED^{441}\downarrow LVV$ ...) in the IGD [6] (see also Figures 4 and 5, fragments 2 and 6).

Detection of AF-28 epitope in MMP digests of whole aggrecan

Whole aggrecan purified from pig laryngeal cartilage was digested with either MMP-8 (Figure 2a) or MMP-1 (Figure 2b) for up to 8 h and aliquots were analysed by composite gel electrophoresis. The results show that undigested aggrecan was not detected with AF-28, confirming the neo-epitope nature of the antibody. AF-28-positive bands could be detected at all subsequent times of digestion. The patterns of AF-28 bands obtained following digestion with the two collagenases were distinctly different from each other and from that of the corresponding Toluidine Blue-stained gels. MMP-8 produced three major and one minor AF-28-positive fragments after 8 h of digestion (Figure 2a, arrows),

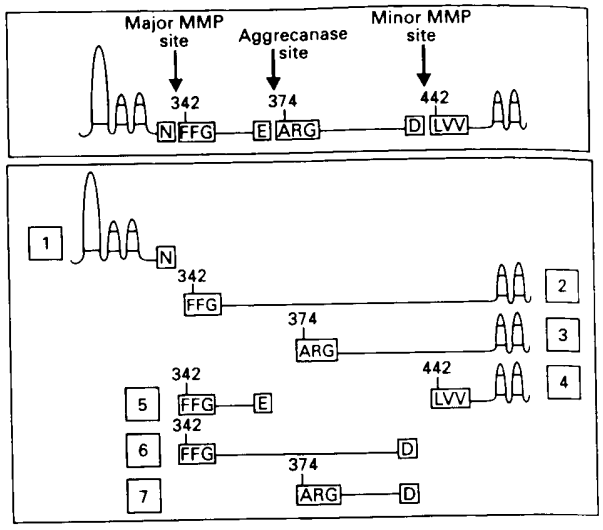

Figure 4 Predicted products of G1–G2 following MMP-8 digestion

Schematic representation of the aggrecan G1–G2 fragment showing the three known MMP-8 cleavage sites [18] and the seven predicted products resulting from cleavage at these sites.

while MMP-1 produced a single major AF-28-positive band after the same time. These results show that AF-28 is a sensitive tool for distinguishing altered patterns of aggrecan degradation *in vitro*.

Detection of AF-28 epitope by competition ELISA

Competitive ELISA experiments provided further evidence that AF-28 was an antibody that recognized a neo-epitope. ELISA plates (96-well) were coated with a 32-mer synthetic peptide with the sequence $F^{342}FGVG ... EGE^{373}$, identical with the sequence in the human aggrecan IGD [17]. Competition experiments were done using (i) FFGVG 5-mer, (ii) DIPENFFGVG 10-mer, (iii) FFGVG ... EGE 32-mer, (iv) FGVGGEEDI 9-mer, (v) undigested aggrecan and (vi) MMP-digested aggrecan as competitors (Figure 3). Competitors with internal ... FFGVG ... sequences (DIPENFFGVG and undigested aggrecan) gave no competition in the assay and maximum antibody binding to the plate was observed at all concentrations of these competitors. The MMP-digested aggrecan and the 32-mer synthetic peptide were equally competitive in the assay on a molar basis (Figure 3a), indicating that the 32-mer was a suitable coating antigen and standard competitor for assaying AF-28 epitope. The results also suggest that keratan sulphate substitution in the IGD does not interfere with detection of the epitope recognized by AF-28, since substituted antigen (aggrecan) and unsubstituted antigen (32-mer) were equally competitive. The FFGVG 5-mer was approx. 50 times less competitive than the 32-mer (Figure 3a), while the FGVGGEEDI 9-mer, which lacked the N-terminal phenylalanine residue, was approximately 230 times less competitive (Figure 3b).

Identification of predicted IGD fragments with AF-28 antibody

We have previously shown that MMP-8 can cleave G1–G2 at the major and minor MMP sites and also at the aggrecanase site [18]. These studies predicted that G1–G2 digested with MMP-8 would produce seven different fragments. Four fragments containing globular G1 or G2 domains (Figure 4, fragments 1–4) have been

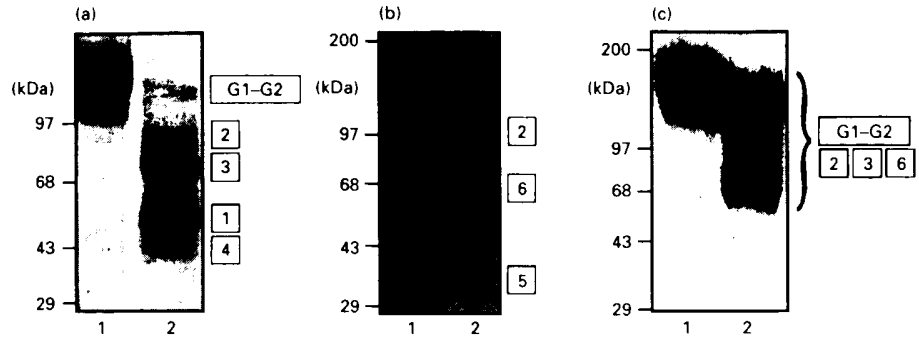

Figure 5 Identification of AF-28-positive IGD fragments

Purified G1–G2 (5 μg) was digested with 160 μg/ml MMP-8 for 21 h, followed by digestion with keratanase. Aliquots of digested and undigested G1–G2 were electrophoresed in triplicate and the bands were detected by (**a**) silver stain, (**b**) AF 28 monoclonal antibody and (**c**) 5-D-4 monoclonal antibody. Numbers to the right of the gels represent the fragments shown in Figure 4. Lanes 1 contain undigested G1–G2.

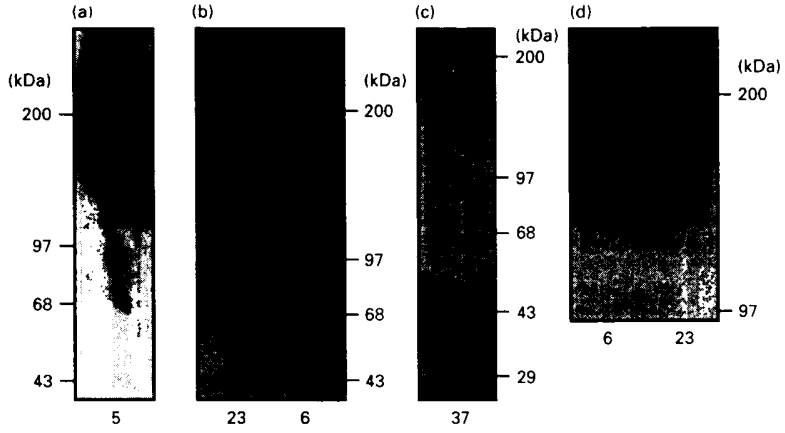

Figure 6 Detection of AF-28 epitope in human synovial fluids

Synovial fluids obtained from patients 5 with rheumatoid arthritis, 23 with juvenile chronic arthritis, 37 with osteoarthritis and 6 with sero-negative inflammatory arthritis were digested with hyaluronidase, electrophoresed on SDS/5%-polyacrylamide gels and analysed for the AF-28 epitope. The gel shown in (**d**) was electrophoresed until the 68 kDa molecular mass marker migrated off the bottom of the gel. This gave better resolution of the bands seen at the top of (**b**).

detected previously by silver staining, immunodetection and N-terminal sequencing [18], but we have not detected the three other IGD fragments lacking globular domains (Figure 4, fragments 5–7).

Cleavage by MMP-8 at the identified sites would produce three fragments with FFGVG... N-terminal sequences as shown in Figure 4, and following Western analysis three bands were detected with AF-28 antibody (Figure 5b, lane 2). The largest fragment detected with AF-28 corresponds in size to fragment 2, which we have characterized and shown to have an FFGVG... N-terminus [18]. The silver-stained gel showed the products previously identified [18]; however, the two faster migrating fragments identified with AF-28 were not readily detected on the silver-stained gel (Figure 5a, lane 2). As there are only three cleavage sites, and fragments 1–4 have previously been identified by sequencing and immunodetection, it is therefore most likely that the two smallest fragments detected by AF-28 antibody correspond to fragments 5 and 6. These fragments, being glycosylated non-globular protein sequences, would not be expected to stain well with silver and this would explain our failure to detect them in previous experiments.

Keratan sulphate chains present on G1–G2 were immunodetected with monoclonal antibody 5-D-4, which recognizes a highly sulphated five disaccharide unit of keratan sulphate [34,35]. The 5-D-4 epitope is resistant to keratanase digestion but can be completely removed by keratanase II digestion. The 5-D-4 antibody detected undigested G1–G2 and a range of digestion products of about 60–150 kDa that were poorly resolved from each other (Figure 5c). The G1 domain and fragment 5 were not detected with 5-D-4.

Identification of AF-28 epitope in human synovial fluids

One of the major reasons for developing a monoclonal antibody with specificity for an FFGVG... neo-epitope was to determine whether MMPs have a role in the *in vivo* degradation of aggrecan. Aggrecan fragments released from cartilage are present in synovial fluids and serum, and numerous studies have measured

protein and carbohydrate epitopes on these fragments. In the present work, aliquots of hyaluronidase-treated human synovial fluids were tested for the presence of AF-28 epitope by competitive ELISA and Western analysis. Of the 40 samples tested, 29 contained detectable levels of AF-28 epitope (range 7.4–52.1 pmol/ml; mean 19.308 ± 9.4 pmol/ml); the results will be published elsewhere. Overnight digestion of synovial fluid samples with chondroitinase ABC, keratanase, keratanase II or 10 μg/ml trypsin did not increase or diminish the level of AF-28 epitope detected in the ELISA, indicating that substituted glycosaminoglycans do not interfere with detection and that trypsin fragments remain large enough to be fully competitive in the assay.

Aliquots of human synovial fluids were electrophoresed on 5% gels in the presence of SDS and analysed for AF-28 epitope (Figure 6). AF-28-positive material was present in broad, ill-defined bands in the molecular mass range 150–250 kDa (Figures 6a and 6b). These high-molecular-mass fragments migrated poorly on 5% gels despite hyaluronidase treatment to lower the viscosity of the fluids; however, if the gels were run until the 68 kDa protein standard ran off the bottom of the gel, the broad smear was resolved into three discrete bands (Figure 6d). Synovial fluids from two patients, one with sero-negative inflammatory arthritis (Figure 6b; patient 3) and the other with osteoarthritis (Figure 6c; patient 4), produced a striking broad band on 5% gels with approximate molecular mass 40–60 kDa that reacted strongly with AF-28 antibody. As reaction with the antibody shows it to have the correct FFGVG... N-terminal sequence, this fragment may represent a more highly glycosylated human equivalent of the F^{342}–E^{373} fragment that was obtained from pig laryngeal aggrecan (Figure 5, fragment 5).

DISCUSSION

We have produced a monoclonal antibody that specifically detects aggrecan fragments produced by MMP cleavage in the IGD. Proteolysis in this region is a key event in aggrecan turnover because it separates the G1 domain, which immobilizes aggrecan by binding to hyaluronan, from the rest of the molecule, which provides the functional weight-bearing properties. The antibody detects an FFGVG... N-terminal epitope present on non-glycosylated polypeptides (molecular mass 3.5 kDa) equally as well as it detects the same N-terminal epitope present on large proteins that are heavily substituted with keratan sulphate and chondroitin sulphate chains. Our results show that AF-28 is a true neo-epitope antibody. It fails to detect internal ... FFGVG... sequences present in undigested G1–G2 and undigested aggrecan by Western analysis. Furthermore, undigested substrates with internal ... FFGVG ... sequences fail to compete in competition assays. In contrast, weak competition can be obtained at high concentrations of peptides containing partial or truncated versions of the epitope (FFGVG 5-mer and FGVGGEEDI 9-mer), provided they are present as N-terminal sequences.

The usefulness of neo-epitope antibodies against aggrecan lies in their potential as diagnostic markers for cartilage destruction. To this end other antibodies that detect aggrecan neo-epitopes have been developed [3,19,20]. Monoclonal CH-3 specifically detects the stromelysin-processed form of cartilage link protein, LP3, but does not detect unprocessed LP1 or LP2 [19]. Monoclonal BC-3 detects N-terminal ARGSV... sequences generated by cleavage at the aggrecanase site [18,20]. Monoclonal [20] and polyclonal [3] antibodies that detect peptides with C-terminal ... DIPEN sequences have also been developed. This ... DIPEN epitope is present on MMP-derived G1 fragments; however, the retention of G1 fragments in mature cartilage [36] limits the usefulness of antibodies with this specificity. The FFGVG... epitope generated by the same MMP action offers greater potential as a marker for aggrecan catabolism because these fragments are not retained in the tissue, but are lost from the matrix into the joint space.

In this paper we show that AF-28 antibody can detect the 32-amino-acid product of combined MMP and aggrecanase activities in pig aggrecan (Figure 5b, fragment 5). To our knowledge, no other antibodies are available which detect this important fragment. We were surprised by the size of the two faster migrating bands detected with AF-28. They were much larger than expected, even allowing for the anomalous behaviour of glycosylated polypeptides on SDS/polyacrylamide gels. Since they contained only 32 and 100 amino acids respectively, the expected molecular sizes of fragments 5 and 6 after deglycosylation were 5–10 kDa for F^{342}–E^{373} and 10–15 kDa for F^{342}–D^{441}. It is clear, however, that residual keratan sulphate chains on these fragments contribute to their apparent increased size even though exhaustive digestion with keratanase did not reduce this size further. It is not clear why the 5-D-4 antibody failed to detect the 32-amino-acid fragment. Whether the 5-D-4 epitope is absent from keratan sulphate chains attached to this sequence or whether a much greater loading of antigen on the gel is necessary to enable detection is not known. Further studies analysing the compositional differences in keratan sulphate chains in the IGD are in progress.

Our data show that the majority of arthritis patients tested in a small pilot study had detectable levels of F^{342}FGVG... epitope in their synovial fluids. In view of other evidence that the majority of aggrecan fragments in human synovial fluids contain A^{374}RGSV... N-termini, our data are compatible with a model which proposes that, *in vivo*, aggrecan processing occurs at both the MMP and the aggrecanase sites. According to this model a large proportion of MMP-derived fragments with FFGVG... N-termini are also aggrecanase-derived fragments with ... ITEGE C-terminal sequences. These fragments comprise 32 amino acids (in the human), contain significant amounts of keratan sulphate [37] and would account for the 40–60 kDa fragment in synovial fluids. Human aggrecan from adult articular cartilage is known to contain significantly more keratan sulphate than aggrecan from young pig laryngeal cartilage [38] and this could explain both the increased size and heterogeneity of the small human fragment compared with the pig fragment. The 40–60 kDa product with an FFGVG... N-terminus is unlikely to have been detected in previous studies that have isolated large, high-density aggrecan fragments with ARGSV... N-termini.

A pathway of aggrecan degradation involving cleavage at both the MMP and the aggrecanase sites is probably not the only route. Indeed, there is evidence for the presence of aggrecan fragments with C-terminal ... $ITEGE^{373}$ sequences in human cartilage [39], suggesting that some fragments have been cleaved at the aggrecanase site yet retain intact ... DIPENFFGVG... sequences. Similarly Vilím and Fosang have shown that at least seven different G1 fragments can be recovered in dissociative extracts of human articular cartilage, indicating that there are multiple catalytic events occurring in human tissue that result in net loss of aggrecan from the tissue [40]. In the absence of quantitative assays for all the relevant epitopes it is not possible to determine the relative abundance of the different neo-epitope products in tissue or fluids.

In summary, monoclonal antibody AF-28 is a sensitive new agent for exploring the complex mechanisms of aggrecan degradation. It is a strict neo-epitope antibody that detects fully glycosylated aggrecan fragments derived from MMP cleavage in the IGD. We have used AF-28 to identify a 32-amino-acid

peptide, F^{342}-E^{373}, in an experimental system and we propose that the F^{342}-E^{373} fragment may also be a product of aggrecan degradation *in vivo*.

Note added in proof (received 7 June 1995).

We wish to acknowledge a recent publication [41] describing a monospecific anti-peptide serum that recognizes the C-terminal sequence of the matrix metalloproteinase-generated aggrecan G1 domain ($VDIPEN^{341}$).

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